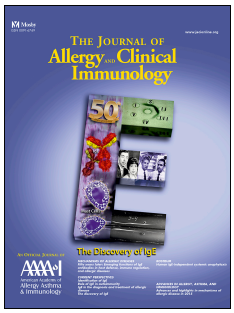
DOI:
10.1016/j.jaci.2017.12.986

Document Version
Peer reviewed version

Accepted Manuscript



Engineering and stable production of recombinant IgE for cancer immunotherapy and AllergoOncology

Silvia Crescioli, PhD, Giulia Chiaruttini, PhD, Silvia Mele, PhD, Kristina M. Ilieva, MRes, Giulia Pellizzari, MSc, Daniel I.R. Spencer, PhD, Richard A. Gardner, PhD, Katie E. Lacy, MD PhD, James F. Spicer, MD PhD, Andrew N.J. Tutt, PhD, Gerd K. Wagner, PhD, Sophia N. Karagiannis, PhD

PII: S0091-6749(18)30081-2

DOI: 10.1016/j.jaci.2017.12.986

Reference: YMAI 13225

To appear in: *Journal of Allergy and Clinical Immunology*

Received Date: 22 July 2017

Revised Date: 9 December 2017

Accepted Date: 18 December 2017

Please cite this article as: Crescioli S, Chiaruttini G, Mele S, Ilieva KM, Pellizzari G, Spencer DIR, Gardner RA, Lacy KE, Spicer JF, Tutt ANJ, Wagner GK, Karagiannis SN, Engineering and stable production of recombinant IgE for cancer immunotherapy and AllergoOncology, *Journal of Allergy and Clinical Immunology* (2018), doi: 10.1016/j.jaci.2017.12.986.

Engineering and stable production of recombinant IgE for cancer immunotherapy and AllergoOncology

Silvia Crescioli PhD[1,2]*, Giulia Chiaruttini PhD[1]*, Silvia Mele PhD[1], Kristina M. Ilieva MRes [1,3] Giulia Pellizzari MSc[1], Daniel I. R. Spencer PhD[4], Richard A. Gardner PhD[4], Katie E. Lacy MD PhD[1], James F. Spicer MD PhD[5], Andrew N. J. Tutt PhD[3,6], Gerd K. Wagner PhD[6], Sophia N. Karagiannis PhD[1,2,3]

[1] St. John's Institute of Dermatology, School of Basic & Medical Biosciences, King's College London, 9th Floor, Tower Wing, Guy's Hospital, London, SE1 9RT, United Kingdom
[2] NIHR Biomedical Research Centre at Guy's and St. Thomas's Hospitals and King's College London, King's College London, London, SE1 9RT, United Kingdom
[3] Breast Cancer Now Unit, School of Cancer & Pharmaceutical Sciences, King`s College London, Guy`s Cancer Centre, London SE1 9RT, United Kingdom
[4] Ludger Ltd, Culham Science Centre, Abingdon, Oxfordshire, OX14 3EB, United Kingdom
[5] School of Cancer & Pharmaceutical Sciences, King`s College London, Guy`s Hospital, Bermondsey Wing, Floor 3, SE1 9RT, London, United Kingdom
[6] Breast Cancer Now Toby Robins Research Centre, Institute of Cancer Research, London, United Kingdom
[7] King's College London, Department of Chemistry, Faculty of Natural & Mathematical Sciences, Britannia House, 7 Trinity Street, London SE1 1DB, United Kingdom

* These authors contributed equally to this work

Corresponding Author:
Sophia N. Karagiannis, PhD, St. John's Institute of Dermatology, School of Basic & Medical Biosciences, King's College London, Guy's Hospital, 9th Floor, Tower Wing, London, SE1 9RT, United Kingdom
E-mail: sophia.karagiannis@kcl.ac.uk
Tel: +44(0)20 7188 6355
Fax: +44(0)20 7188 8050

Acknowledgements
The research was supported by the National Institute for Health Research (NIHR) BRC based at Guy's and St Thomas' NHS Foundation Trust and King's College London (IS-BRC-1215-20006). The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR or the Department of Health. The authors acknowledge support by the Medical Research Council (MR/L023091/1); Breast Cancer Now (147), working in partnership with Walk the Walk; The British Skin Foundation (S633); The Dermatrust; Guy's and St Thomas's Charity Melanoma Special Fund; Cancer Research UK (C30122/A11527; C30122/A157745); the Academy of Medical Sciences; CR UK/EPSRC/MRC/NIHR KCL/UCL Comprehensive Cancer Imaging Centre (C1519/A10331); CR UK/NIHR in England/DoH for Scotland, Wales and Northern Ireland Experimental Cancer Medicine Centre (C10355/A15587); BBSRC IBCarb Network (Proof-of-Concept award IBCarb-PoC-0616-040). We acknowledge the Biomedical Research Centre Immune Monitoring Core Facility team at Guy's and St Thomas' NHS Foundation Trust for assistance.

Capsule summary

We developed a versatile, novel, time- and resource-effective tool for production of functional IgE at high yields that has wide application potential in basic research and clinical evaluations in allergy, cancer immunotherapy and AllergoOncology.

Key words

AllergoOncology; IgE; cancer immunotherapy; effector cells; degranulation; antibody engineering

Abbreviations

Human Embryonic Kidney (HEK), Chinese Hamster Ovary (CHO), Polymerase Incomplete Primer Extension (PIPE), Ubiquitous Chromatin Opening Elements (UCOE), chondroitin sulphate proteoglycan 4 (CSPG4), good manufacturing practice (GMP), Tumor Associated Antigen (TAA), Enzyme-linked immunosorbent assay (ELISA), High Pressure Liquid Chromatography (HPLC), Ricinus Cummunis Agglutinin I (RCAI), Aleuria Aurantia lectin (AAL), Concavalin A (ConA).

Engineering and stable production of recombinant IgE for cancer immunotherapy and AllergoOncology

To the Editor:

AllergoOncology, the emerging discipline of cancer immunology aiming to exploit features of allergy-related immunity to treat tumors (1–3), has catalyzed the development of tumor-specific IgE monoclonal antibodies as powerful alternatives to commonly-used therapeutic IgGs (4–6). IgE typically associated with the pathogenesis of allergic responses and known for Fc-mediated protective effects in parasitic infection clearance, presents exciting opportunities to unleash previously-untapped immune mechanisms and effective anti-tumor surveillance when focused against cancer antigens. Anti-tumor efficacy of IgE has been demonstrated by numerous studies (1–3) and an early clinical trial of the first-in-class anti-tumor IgE in oncology is open (NCT02546921, www.clinicaltrials.gov). A major impediment in the field relates to lack of efficient cloning and production strategies for recombinant IgE at high-enough yields for pre-clinical and clinical studies.

We aimed to develop a stable expression application to generate recombinant IgE, exemplified with an antibody recognizing the melanoma-associated antigen chondroitin sulphate proteoglycan 4 (CSPG4). Our strategy incorporates seamless cloning, selection and fast antibody production at high yields (Fig. 1a). To prevent promoter silencing, we developed a novel dual-plasmid system containing Ubiquitous Chromatin Opening Elements (UCOE) sequences, located upstream of the transgene promoter (7). We isolated the coding sequences of anti-CSPG4 IgE heavy and light chains from a previously-described (pVITRO1-CSPG4 IgE/k) vector (4) (Fig. 1b) and cloned these into two UCOE-vectors (UCOE-CSPG4-HC(ε), UCOE-CSPG4-LC(κ)) (Fig. 1c), employing Polymerase Incomplete Primer Extension (PIPE) cloning. UCOE enables higher transfection efficiency and higher proportions of medium- and high-expressing transfectomas than pVITRO1 (see Figure E1 in the Online Repository). Vectors were linearized before transfections to allow correct integration into the host genome, and transgene-expressing cells were selected. The choice of Expi293F^{TM} as hosts was based on human-like glycosylation profiles, ability to grow in suspension, high-density and serum-free conditions, characteristics crucial for expediting production, scaling up and adaptability to good manufacturing practice (GMP) conditions. We adapted Expi293F^{TM} cells from suspension to adherent growth

conditions and vice-versa. Adherent cells were transfected and seeded in selection medium to promote host genome integration of exogenous DNA. Resistant cells were cloned by limiting dilution. We designed a cell-based flow cytometric method to detect functional IgE recognizing natively-expressed antigens to screen antibody-secreting clones (see Figure E1 in the Online Repository). High antibody-expressing clones were amplified and re-adapted to grow in high-density suspension cultures for antibody harvesting.

After selecting the highest-expressing clone, we optimized culture conditions to maximize IgE production and minimize time and resources. We observed a slow decrease of specific daily antibody productivity, consistent with cell growth rate and consumption of culture medium nutrients. This productivity decrease was due to nutrient depletion in the medium rather than cell density (see Figure E2 in the Online Repository). To maximize yields, we tested different seeding Expi-CSPG4 IgE cell concentrations in fresh medium, measuring secreted antibody daily for 5 days. As expected, higher starting cell concentration yielded faster and higher antibody production, with cells seeded at $11x10^6$ cells/mL generating 2mg/day (Fig. 1d).

Using high cell concentrations ($5x10^6$ and $11x10^6$ cells/mL), that place cells under stress, we analysed production consistency over time by passaging every two days at $5x10^6$ (5M/mL 2D), or every day at $5x10^6$ (5M/mL 1D) or $11x10^6$ cells/mL (11M/mL 1D), replacing media at every passage. After 4 days all conditions yielded consistent antibody production (Fig. 1e). The 11M/mL 1D and 5M/mL 2D conditions yielded similar production per passage. However, 11M/mL 1D resulted in the highest production per day (see Figure E3 in the Online Repository), suggesting this is optimal for reducing resources and time.

IgE production reached yields of up to 87mg/L/day (83±4 mg/L/day mean±SD), with ability to repeat the process with the same cells at least 3 times without losing production efficiency. Yields in 4 days, in small shaking flask cultures, were approximately 33-fold higher than the most optimal 14-day stable IgE production recorded in shaking flask conditions, and 13-fold higher than 14-day IgE production reported using bioreactors (4). Optimized high-density conditions allowed maximized yields and substantially-reduced medium volumes, achieving 2mg/25mL/day, with similar yields scaling down to 15mL and up to 300mL cultures.

Different culture conditions can affect antibody quality, structural and functional properties, including post-translational modifications such as glycosylation (8). This is highly pertinent for IgE, based on larger size and higher glycosylation levels compared with IgG. We performed structural, glycosylation and functional analyses comparing affinity chromatography-purified antibodies from high- and low-density cultures alongside IgE produced with the previous pVITRO method (4).

Size-exclusion High Pressure Liquid Chromatography (HPLC), showed very similar antibody main peak profiles (Fig. 2a). Lectin blot and LC-MS glycosylation analyses, revealed no significant differences among IgE produced with our method (Fig. 2b, 2c), particularly with regards to oligomannose structures, whose removal is reported to abrogate anaphylaxis (9). LC-MS glycosylation analyses showed a reduction in MAN5 oligomannose structure in new preparations compared to pVITRO IgE. Antibodies from all conditions showed comparable binding characteristics to target antigen on A375 melanoma cells and to rat basophilic leukemia RBL-SX38 cells expressing human FcεRI (Fig. 2d). All preparations triggered significant and comparable levels of mast cell degranulation when cross-linked by polyclonal anti-IgE or by target antigen on CSPG4^{high} melanoma cells (Fig. 2e). The hapten-specific NIP-IgE cross-linked by polyclonal anti-IgE, but not by CSPG4^{high} cells, triggered significant degranulation. These suggest that different preparations and density conditions preserve receptor recognition and antibody potency. Importantly, reduced MAN5 in the new IgE is insufficient to impact IgE binding or functionality.

We therefore present a novel process for serum-free production of IgE with comparable structural and functional characteristics to previous pVITRO method, but at higher yield and in less time than any documented stable platforms and with less resources than transient systems (see Table E1 in the Online Repository). This offers new opportunities to expedite the design of novel therapeutic antibodies with enhanced effector functions, suitable for basic and translational research, scale up, process development and manufacturing for clinical trials of IgE therapy in cancer. Rapid generation of IgE antibodies recognizing allergens, cancer antigens or parasitic targets can find direct applications for exploring multifaceted functions of IgE in Allergy, Oncology and AllergoOncology.

Silvia Crescioli PhD[1,2]*, Giulia Chiaruttini PhD[1]*, Silvia Mele PhD[1], Kristina M. Ilieva MRes [1,3] Giulia Pellizzari MSc[1], Daniel I. R. Spencer PhD[4], Richard A. Gardner PhD[4], Katie E. Lacy PhD[1], James F. Spicer PhD[5], Andrew N. J. Tutt PhD[3,6], Gerd K. Wagner PhD[6], Sophia N. Karagiannis PhD[1,2,3]

[1] St. John's Institute of Dermatology, School of Basic & Medical Biosciences, King's College London, 9th Floor, Tower Wing, Guy's Hospital, London, SE1 9RT, United Kingdom
[2] NIHR Biomedical Research Centre at Guy's and St. Thomas's Hospitals and King's College London, King's College London, London, SE1 9RT, United Kingdom
[3] Breast Cancer Now Unit, School of Cancer & Pharmaceutical Sciences, King`s College London, Guy`s Cancer Centre, London SE1 9RT, United Kingdom
[4] Ludger Ltd, Culham Science Centre, Abingdon, Oxfordshire, OX14 3EB, United Kingdom
[5] School of Cancer & Pharmaceutical Sciences, King`s College London, Guy`s Hospital, Bermondsey Wing, Floor 3, SE1 9RT, London, United Kingdom
[6] Breast Cancer Now Toby Robins Research Centre, Institute of Cancer Research, London, United Kingdom
[7] King's College London, Department of Chemistry, Faculty of Natural & Mathematical Sciences, Britannia House, 7 Trinity Street, London SE1 1DB, United Kingdom

* These authors contributed equally to this work

Corresponding Author:
Sophia N. Karagiannis, PhD, St. John's Institute of Dermatology, School of Basic & Medical Biosciences, King's College London, Guy's Hospital, 9th Floor, Tower Wing, London, SE1 9RT, United Kingdom
E-mail: sophia.karagiannis@kcl.ac.uk
Tel: +44(0)20 7188 6355
Fax: +44(0)20 7188 8050

Author contributions

S.N.K., S.C. and G.C. conceived and designed the study. S.M. K.M.I. and G.P. helped with the development of the methodology. S.C. and G.C. designed, performed the experiments and analyzed the data. D.I.R.S. and R.A.G. carried out the glyco-analysis and analysed the data, with contributions from G.K.W., S.N.K., S.C., G.C., S.M., K.M.I., G.P., D.I.R.S., R.A.G., G.W., J.F.S., A.N.T. and K.E.L. All authors discussed and interpreted data and edited the manuscript. S.N.K. led and supervised the study and coordinated the project. S.C., G.C., and S.N.K. wrote the manuscript.

Competing financial interests

S.N.K. and J.F.S. are founders and shareholders of IGEM Ltd. S.N.K. holds a registered patent for anti-CSPG4 IgE antibodies. All other authors declare no conflicts of interest.

Figure Legends

Figure 1. Development of a stable platform for the expression of recombinant IgE (a) Flow chart summarising the development of stable cell lines expressing anti-CSPG4 IgE. (**b)** pVITRO1-CSPG4-IgE/κ vector and **(c)** UCOE-CSPG4-HC(ε) and UCOE-CSPG4-LC(κ) vectors maps. To optimize antibody production Expi-CSPG4-IgE cells were cultured in different conditions and IgE secretion and cell viability were monitored daily. (**d**) Secreted IgE in cultures seeded at 0.5, 2, 5 or $11x10^6$ cells/mL in fresh medium. (**e**) Secreted IgE in cultures seeded at $5x10^6$ or $11x10^6$ cells/mL in 25 mL fresh medium and re-seeded at the initial concentration every day (5M/mL 1D, 11M/mL 1D) or every two days (5M/mL 2D). (d, e) Data represent mean ± SEM of four independent experiments.

Figure 2. Structural and functional characterization of IgE produced under different conditions. IgEs were purified from supernatant of Freestyle293F-CSPG4 IgE (pVITRO) or Expi-CSPG4 IgE cultured at $0.5x10^6$ for 4 days, (0.5M/mL 4D), $5x10^6$ for 2 days (5M/mL 2D), $5x10^6$ for 1 day (5M/mL 1D) or $11x10^6$ cells/mL for 1 day (11M/mL 1D). Structural characterization was performed by (**a**) HPLC; (**b**) Lectin blot using Con-A, AAL, and RCAI. Images show one representative experiment. Graphs show densitometric analyses normalized by kappa light chain western blot. Data represent mean ± SEM of two independent experiments. (**c**) LC-MS glycosylation analysis, graph represents the relative % areas for each UHPLC chromatograms peak of procainamide labelled N-glycan released from each sample. Data represent mean ± SD. Monosaccharide compositions assigned to peaks: H=hexose, N=N-acetylhexosamine, F=fucose, S=sialic acid; Possible N-glycan structures shown in brackets: F=Fucose, A=antenna, G=galactose, MAN=mannose, S=sialic acid. (**d**) IgE binding kinetics to CSPG4 antigen (A375 cells) and FcεRI (RBL-SX38 cells). (**e**) IgE mediated degranulation of RBL-SX38 mast cells, measured in: negative control (no cross-linker), positive control (polyclonal anti-IgE), and using a CSPG4-expressing tumour cell line to trigger cross-linking of anti-CSPG4 IgE-FcεRI complexes. Data represent mean ± SEM of four independent experiments (ns = not significant, ** = $P<0.01$, **** = $P<0.0001$).

References

1. Josephs DH, Bax HJ, Dodev T, Georgouli M, Nakamura M, Pellizzari G, et al. Anti-Folate Receptor-α IgE but not IgG Recruits Macrophages to Attack Tumors via TNFα/MCP-1 Signaling. Cancer Res. 2017 Mar 1;77(5):1127–41.

2. Jensen-Jarolim E, Bax HJ, Bianchini R, Capron M, Corrigan C, Castells M, et al. AllergoOncology - the impact of allergy in oncology: EAACI position paper. Allergy. 2017 Jun;72(6):866–87.

3. Jensen-Jarolim E, Turner MC, Karagiannis SN. AllergoOncology: IgE- and IgG4-mediated immune mechanisms linking allergy with cancer and their translational implications. J Allergy Clin Immunol. 2017 May 17;

4. Dodev TS, Karagiannis P, Gilbert AE, Josephs DH, Bowen H, James LK, et al. A tool kit for rapid cloning and expression of recombinant antibodies. Sci Rep. 2014 Jul 30;4(1):5885.

5. Bantleon F, Wolf S, Seismann H, Dam S, Lorentzen A, Miehe M, et al. Human IgE is efficiently produced in glycosylated and biologically active form in lepidopteran cells. Mol Immunol. 2016 Apr;72:49–56.

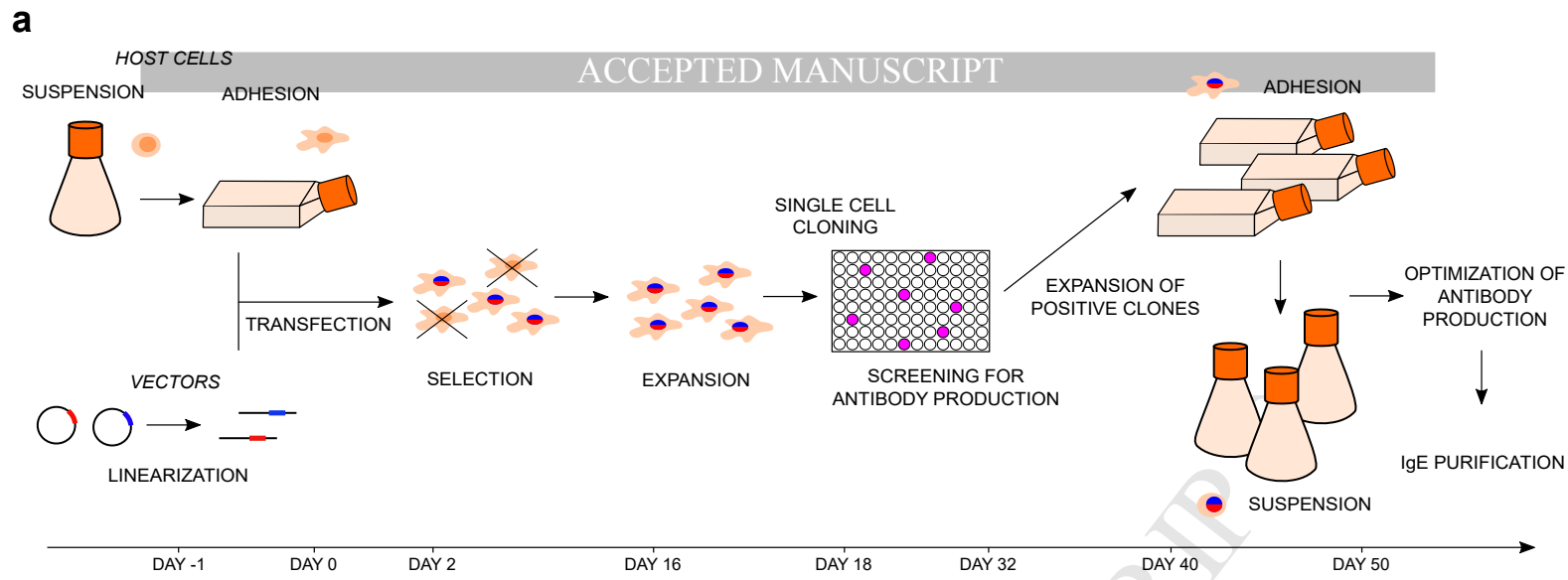
6. Montero-Morales L, Maresch D, Castilho A, Turupcu A, Ilieva KM, Crescioli S, et al. Recombinant plant-derived human IgE glycoproteomics. J Proteomics. 2017 May 24;161:81–7.

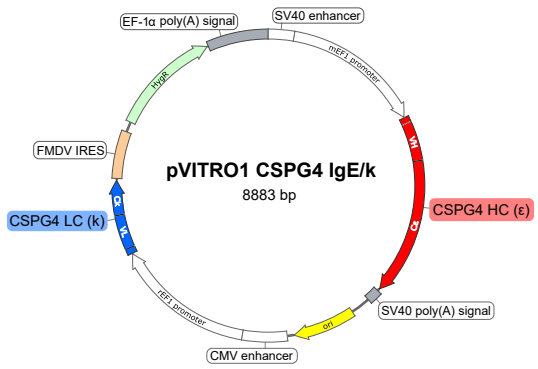
7. Puchacz E, Spanggord R, Shelton U. An Accelerated Method for Production of Recombinant Proteins using UCOE™ Expression Technology. Nature Methods | Application Notes. 2007 Dec 11;

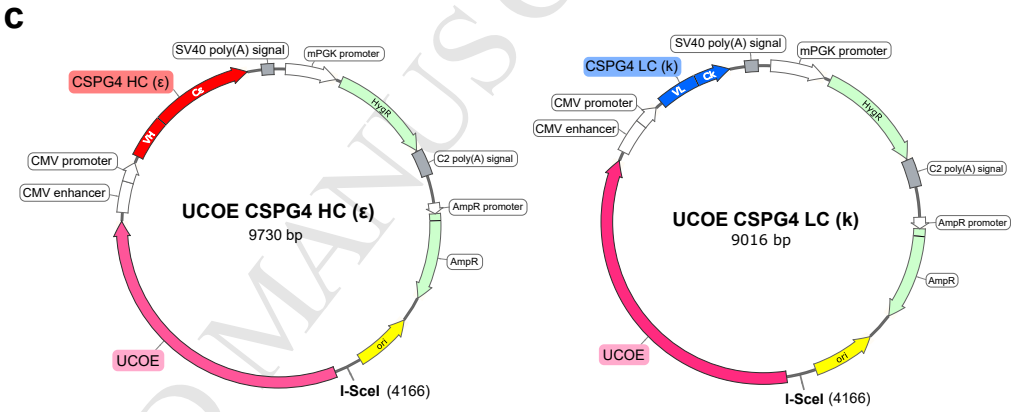
8. Ivarsson M, Villiger TK, Morbidelli M, Soos M. Evaluating the impact of cell culture process parameters on monoclonal antibody N-glycosylation. J Biotechnol. 2014 Oct 20;188:88–96.

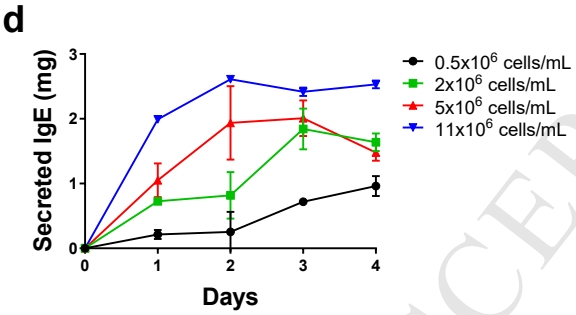
9. Shade K-TC, Platzer B, Washburn N, Mani V, Bartsch YC, Conroy M, et al. A single glycan on IgE is indispensable for initiation of anaphylaxis. J Exp Med. 2015 Apr 6;212(4):457–67.

Figure 1









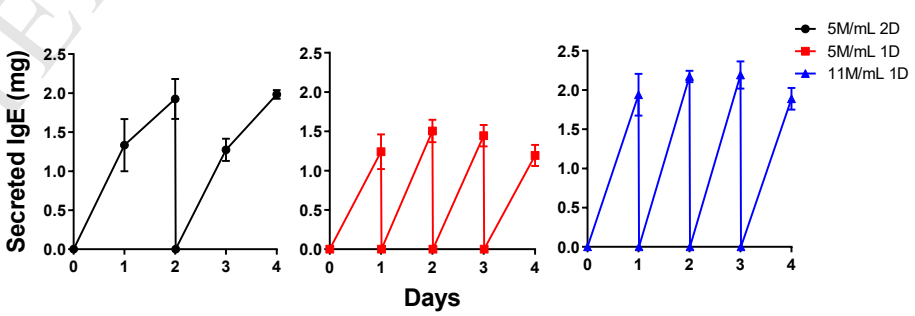
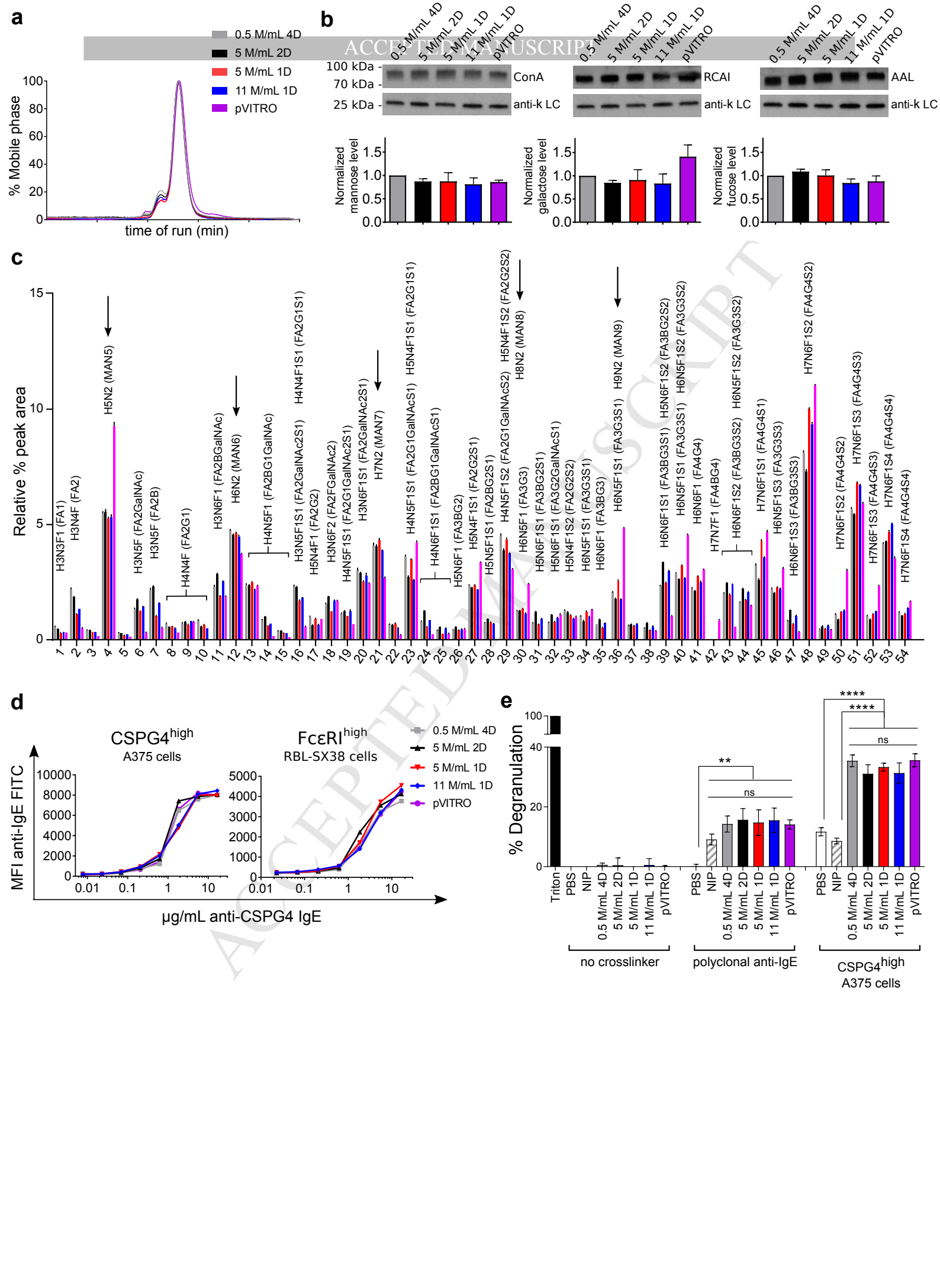


Figure 2



Materials and Methods

Cell lines

Expi293F™ were cultured in Expi293™ Expression Medium in suspension and shaking conditions (125rpm) at 37°C and 8% CO_2 according to the manufacturer's instructions, seeding them at $0.5x10^6$ cells/mL every 4 days. A375 melanoma cells were cultured in adhesion in Dulbecco's modified Eagle's medium (DMEM) high glucose supplemented with GlutaMAX™ (Gibco) and 10% Fetal Bovine Serum (FBS). RBL-SX38 rat basophilic leukemia cells (engineered to express the human FcεRI) (1) were cultured in adhesion.

CSPG4 IgE expression vectors

The pVITRO1-CSPG4-IgE/k vector (Fig 1b) was previously developed in our group (2). The dual UCOE-vector system (UCOE-CSPG4-HC(ε) and UCOE-CSPG4-LC(κ)) (Fig 1c) was developed by Polymerase Incomplete Primer Extension (PIPE) cloning as described before (2). Briefly, the coding sequence of CSPG4 IgE heavy and light chains were isolated from pVITRO1-CSPG4-IgE/k vector and UCOE® Mu-H vector (Merck Millipore) was linearized in three portions by using the primers in Table E2 and the PCR conditions reported in Table E3 in the Online Repository. The PCR products were then digested for 2 hours at 37°C with DpnI (NEB) to eliminate template DNA, and two separate mixes were prepared using the three vector portions and one heavy or light chain. The two mixes were incubated overnight at room temperature and then used to transform chemically competent One Shot™ Top10 bacteria (Invitrogen) according to the manufacturer's instruction.

Development of stable IgE expressing Expi293F™ cells

The Expi293F™ cell line (Gibco) was cultured according to the manufacturer's instructions in Expi293™ Expression Medium (Gibco). One day before transfection, $2x10^5$ cells were seeded in a 24-well plate in DMEM high glucose supplemented with GlutaMAX™ (Gibco) and 10% FBS in order to simultaneously adapt them to grow in adherence conditions and achieve 90% confluence at the point of transfection. UCOE-

CSPG4-HC(ε) and UCOE-CSPG4-LC(k) were linearized using I-SceI (NEB) according to the manufacturer's instruction. Adherent cells were transfected with either pVITRO1-CSPG4-IgE/k or the two linearized UCOE-vectors, using 0.8µg of DNA, 2µL of Lipofectamine® 2000 (Invitrogen) and 600µL of Opti-MEM® I Reduced Serum Medium (Gibco) according to the manufacturer's instructions. A mock transfection (without vector) was performed as control. Sixteen hours after the transfection the cells were split 1:10 and seeded in complete medium (DMEM-high glucose supplemented with GlutaMAX^{TM} (Gibco) and 10% FBS). The following day media were replaced with selection medium (complete medium supplemented with 200µg/mL Hygromycin B (Sigma)). Cells were then cultured replacing the selective medium every 3 days until the point when all the cells from the mock transfection died. The selection-resistant cells were then amplified and cloned by limiting dilution in selection media. After 2 weeks, the clones were screened for the secretion of anti-CSPG4 IgE in the supernatant, positive clones were amplified and then adapted back to growth in suspension and shaking conditions in Expi293^{TM} Expression Medium. Different clones were frozen and the best clone (Expi-CSPG4 IgE) was used for the optimization of antibody production.

CSPG4 IgE production with stable Freestyle293-CSPG4 IgE cells

CSPG4 IgE was produced culturing Freestyle293-CSPG4 IgE according to our previously described method (pVITRO1)(2).

ELISA assay for the detection and quantification of IgE in cell supernatants

To quantify anti-CSPG4 IgE in cell culture supernatants, 96-well plates were coated overnight at 4°C with 10µg/mL of polyclonal rabbit anti-IgE (Dako) in 0.2M carbonate-bicarbonate buffer, pH 9.4. Plates were incubated in blocking buffer (Phosphate buffered saline (PBS) (0.01M phosphate buffer, 0.0027M KCl, 0.14M NaCl) pH 7.4 supplemented with 1% BSA) for 1 hour at room temperature, washed 3 times with washing buffer (PBS+0.05% Tween-20) and incubated with supernatants or with the IgE antibody standard at different dilutions for 2 hour at room temperature. All conditions were tested in duplicate. Plates were washed 3 times and incubated with 10µg/mL goat anti-human Fcε-HRP (Sigma-Aldrich) in blocking

buffer for 1.5 hours at room temperature. Following 3 washes, samples were treated with 50µL of 0.5mg/mL o-phenylenediaminedihydrochloride substrate (Sigma-Aldrich) in peroxide substrate buffer (Pierce) for 5 minutes at room temperature. Following addition of 50µL per well Stop Solution (1M HCl), plates were read at 492nm (reference wavelength 540nm) on a Fluostar Omega microplate reader (BMG Labtech).

Flow cytometry-based assay for the detection and quantification of antigen-specific IgEs in cell supernatants

This method is based on the detection, in cell supernatants, of IgE antibodies that specifically bind to CSPG4^{high} tumor (A375 melanoma) cells. A375 cells were detached using PBS supplemented with 5mM EDTA and resuspended in PBS supplemented with 2% FBS (FACS buffer). For this assay were used 10^5 cells per sample in a final volume of 100 µL. For the quantification of antigen-specific IgE a standard curve was prepared using 1:2 serial dilutions of CSPG4 IgE. For the analysis of CSPG4 IgE in cell culture supernatants, 0.1µL of supernatant was prepared FACS buffer, for a final dilution of 1:1,000. Primary antibodies were incubated 30 minutes at 4 °C followed by a wash with 3 mL of FACS buffer (spinning at 500g for 5 minutes). The secondary antibody goat anti-human IgE FITC (FI-3040, Vector Laboratories) was incubated at 30µg/mL in FACS buffer for 30 minutes at 4 °C followed by one wash as above. Samples were resuspended in 100µL of FACS buffer and analysed using a FACS Canto II (BD Biosciences).

Purification of CSPG4 IgE

CPSG4 IgE was purified using HiTrap KappaSelect columns (GE Healthcare) according to the manufacturer's instructions. Briefly, columns were equilibrated with 10 column volumes (cv) of PBS, cell culture supernatant was diluted 1:1 v/v with PBS and samples were loaded on the column followed by a wash using at least 20 cv of PBS. The sample was then eluted with 5 cv of 0.1M glycine buffer, pH 2.3 and immediately buffered to pH 7.5 using 1 M Tris, pH 9.0. Purified antibodies were then dialyzed against PBS overnight at 4 °C and sterilized using a 0.2µm sterile filter.

Size exclusion chromatography

Purified antibodies were analysed by size exclusion chromatography as previously described (3). Briefly, gel filtration was performed on a Gilson HPLC system using a Superdex^{TM} 200 10/300 GL column (GE Healthcare), suitable for purifying proteins between 10–300 kDa, at a flow rate of 0.75 mL/min in PBS (pH 7.0, 0.2μm filtered).

Lectin blot

Purified IgE samples (150ng) were reduced with 50 mM DTT and boiled at 95 °C for 5 minutes. The samples were run at 150V on Mini-PROTEAN TGX Gels 4–15% (Bio-Rad) and blotted with Trans-Blot® Turbo™ Transfer Pack PVDF (Bio-Rad) using Trans-Blot® Turbo™ Blotting System (Bio-Rad) according to manufacturer's instruction. The blotted membrane was then cut just above 35kDa in order to have heavy (50kDa) and light (25kDa) chain in different membranes. The heavy chain membrane was blocked with Carbo-Free™ Blocking Solution (Vectorlab) for 1 hour and then probed with RCAI-biotin (Ricinus Cummunis Agglutinin I lectin (Vectorlab) specific for Galactose), or AAL-biotin (Aleuria Aurantia lectin (Vectorlab) specific for Fucose), or Con A-biotin (Concavalin A lectin (Vectorlab) specific for Mannose), 0.2μg/mL in Carbo-Free™ Blocking Solution for 30 minutes. The membrane was then washed three times in PBS-Tween 0.05% (T-PBS) and incubated with High Sensitivity HRP-streptavidin (Pierce) (1:30,000) for 30 minutes, washed as above and developed with ECL (Amersham, GE Healthcare). The light chain membrane was blocked with T-PBS 5% BSA for 1 hour at room temperature and then incubated overnight at 4°C with rabbit anti-human-kappa light chain antibody (Abcam) (1:1,000 in T-PBS 5% BSA) followed by three washes in T-PBS. The membrane was incubated with anti-rabbit-IgG HRP antibody (Cell Signaling Technology) (1:2,000 in T-PBS 5% BSA) for 1 hour at room temperature, washed as above and developed with ECL. Densitometric quantification was performed with ImageJ software, values were normalized by the loading control (kappa light chain) and for the value obtained in 0.5M/mL 4D culture condition.

PNGase F release of N-glycans

IgE samples weredried down prior to releaseThe PNGase F release of N-glycans (E-PNG01, QABio) was performed in a similar manner to that previously published (4). In short, 25 µg aliquots of the samples in water (17.5 µL) were incubated with 5 µL of 5x Reaction Buffer 7.5 and 1.25 µL of Denaturation Solution for 10 minutes at 100°C followed by the addition of Triton X-100(1.25 µL) and PNGase F solution (1 µL) and incubation overnight at 37°C. Dried-down samples were treated with 20 µL of 1% formic acid for 50 minutes at RT followed by removal of protein material using a Protein Binding Membrane (PBM) plate (LC-PBM-96, Ludger Ltd).

Procainamide labeling and cleanup

Released glycans were labelled with procainamide using a procainamide labelling kit (LT-KPROC-24, Ludger Ltd) in a similar manner to that previously reported (5). In short, 150 µL of 30% glacial acetic acid in DMSO was added to a vial of procainamide followed by 150 µL of water. This solution was transferred to a vial of sodium cyanoborohydride to make the final labelling reagent. 20 µL of labeling reagent was added to each sample. The samples were then incubated for 1 hour at 65°C. A HILIC method was performed to clean up the samples and remove free dye using an LC-PROC-96 plate (Ludger Ltd) on a vacuum manifold. Samples were eluted from the cleanup plate in 300 µL of water.

LC-MS analysis of procainamide labeled N-glycans

Samples were analysed by HILIC-LC on an Ultimate 3000 UHPLC using a BEH-Glycan 1.7µm, 2.1x150mm column (Waters) at 40°C with a fluorescence detector (λex = 310nm, λem = 370nm), controlled by Bruker HyStar 3.2 (Bruker). Buffer A was 50mM ammonium formate made from LudgerSep N Buffer stock solution, pH 4.4 [LS-N-BUFFX40]; buffer B was acetonitrile (acetonitrile 190 far UV/gradient quality; Romil #H049). Samples were injected in 25% aqueous/75% acetonitrile; injection volume 25 µL. The separation was performed using a linear gradient of 76-53% ACN at 0.4mL/min in a 71 minute analytical run. PROC labelled glucose homopolymer was used as a system suitability standard as well as an external calibration standard for GU allocation for the system. Chromeleon data software version 7.2 (Thermo) with a cubic spline fit was used to allocate glucose unit (GU)

values to peaks. A Bruker amaZon Speed ETD electrospray mass spectrometer (Bruker) was coupled directly after the UHPLC FD without splitting. The instrument scanned samples in maximum resolution mode, positive ion setting, MS scan + three MS/MS scans, nebuliser pressure 14.5 psi, nitrogen flow 10L/min, capillary voltage 4500 Volts. MS/MS was performed on three ions in each scan sweep with a mixing time of 40 ms. Mass spectrometry data were analysed using the Bruker Compass DataAnalysis 4.1 software.

Binding of CSPG4 IgE to tumor and immune cell lines

Binding of CSPG4 IgE to A375 and RBL SX38 cells was performed using 10^5 cells per sample. Cells were detached using PBS + 5mM EDTA. For each primary antibody, serial dilutions were prepared in FACS buffer (PBS, 2% FBS). Primary antibodies were incubated for 30 minutes at 4°C followed by a wash with 3 mL of FACS buffer (spinning at 500g for 5 minutes). Samples were incubated with secondary antibody goat anti-human IgE FITC (FI-3040, Vector Laboratories) at 30 µg/mL in FACS buffer per sample for 30 minutes at 4°C followed by one wash as above. Samples were resuspended in 100µL of FACS buffer and analysed using a FACS Canto II.

Rat basophilic leukaemia SX-38 degranulation assays

We evaluated degranulation of RBL-SX38 cells by quantifying b-hexosaminidase release, as previously described (6,7). Control conditions used were: unstimulated cells; Triton X-100 lysed cells (to quantify 100% granule release); chimeric NIP IgE against the hapten 5-iodo-4-hydroxy-3-nitrophenyl; Unstimulated or NIP IgE-stimulated cells plus polyclonal rabbit anti-IgE (Dako) or antigen-expressing tumor cells as cross-linkers. Cells were seeded at $1x10^4$ cells per well in culture medium overnight. The next day cells were sensitized with IgE (200ng/mL), control antibody or medium alone by incubating them at 37°C for 2 hours. Cells were then washed 3 times in stimulation buffer (HBSS, 1% FBS; Gibco) and stimulated for 45 minutes at 37°C with 100µL stimulation buffer, rabbit anti-IgE (1.5µg/mL), or $3x10^4$ $CSPG4^{high}$ A375 tumour cell line. To detect β-hexosaminidase, 50µL culture supernatants diluted 1:1 in stimulation buffer were transferred onto black 96-well plates with 50µL

florigenic substrate per well (1mM 4-methylumbelliferyl N-acetyl-b-D-glucosaminide, 0.1% DMSO, 0.1% Triton X-100, 200mM citrate, pH 4.5) and incubated for 2 hours in the dark at 37°C. Reactions were quenched with 100µL per well 0.5 M Tris and plates read with a Fluostar Omega microplate reader (350 nm excitation, 450 nm emission) (BMG Labtech). Degranulation was expressed as a percentage of Triton X-100 release (100%), and compared with unstimulated cells (0%).

Evaluation of antibody-specific productivity

Expi-CSPG4-IgE cells were cultured in different conditions and IgE secretion and cell viability were monitored daily. Antibody-specific productivity q_{mAb} (pg cell^{-1} day^{-1}) was calculated according to the following equation (8):

$$q_{mAb} = \frac{m_{mAb}}{\frac{(N - N_0) \times t}{\log_e(N/N_0)}}$$

with m_{mAb} being secreted IgE, N and N_0 being the final and the initial viable cell values, respectively, and t being the days in culture.

Statistical analysis

Error bars represent the standard deviation (SD) or the standard error of the mean (SEM). The statistical significance of degranulation assay was calculated with one-way ANOVA with Dunnett's multiple comparisons test. P values lower than 0.05 were considered statistically significant, single, double, triple and quadruple asterisks indicate $P < 0.05$, $P < 0.01$, $P < 0.001$, $P < 0.0001$, respectively. Data were analyzed with Prism 7 software (GraphPad).

Bibliography

1. Wiegand TW, Williams PB, Dreskin SC, Jouvin MH, Kinet JP, Tasset D. High-affinity oligonucleotide ligands to human IgE inhibit binding to Fc epsilon receptor I. J Immunol. 1996 Jul 1;157(1):221–230.

2. Dodev TS, Karagiannis P, Gilbert AE, Josephs DH, Bowen H, James LK, et al. A tool kit for rapid cloning and expression of recombinant antibodies. Sci Rep. 2014 Jul 30;4(1):5885.

3. Hunt J, Beavil RL, Calvert RA, Gould HJ, Sutton BJ, Beavil AJ. Disulfide linkage controls the affinity and stoichiometry of IgE Fcepsilon3-4 binding to FcepsilonRI. J Biol Chem. 2005 Apr 29;280(17):16808–16814.

4. Ventham NT, Gardner RA, Kennedy NA, Shubhakar A, Kalla R, Nimmo ER, et al. Changes to serum sample tube and processing methodology does not cause Intra-Individual [corrected] variation in automated whole serum N-glycan profiling in health and disease. PLoS ONE. 2015 Apr 1;10(4):e0123028.

5. Kozak RP, Tortosa CB, Fernandes DL, Spencer DIR. Comparison of procainamide and 2-aminobenzamide labeling for profiling and identification of glycans by liquid chromatography with fluorescence detection coupled to electrospray ionization-mass spectrometry. Anal Biochem. 2015 Oct 1;486:38–40.

6. Dibbern DA, Palmer GW, Williams PB, Bock SA, Dreskin SC. RBL cells expressing human FcεRI are a sensitive tool for exploring functional IgE–allergen interactions: studies with sera from peanut-sensitive patients. J Immunol Methods. 2003 Mar;274(1-2):37–45.

7. Karagiannis P, Singer J, Hunt J, Gan SKE, Rudman SM, Mechtcheriakova D, et al. Characterisation of an engineered trastuzumab IgE antibody and effector cell mechanisms targeting HER2/neu-positive tumour cells. Cancer Immunol Immunother. 2009 Jun;58(6):915–930.

8. Chusainow J, Yang YS, Yeo JHM, Toh PC, Asvadi P, Wong NSC, et al. A study of monoclonal antibody-producing CHO cell lines: what makes a stable high producer? Biotechnol Bioeng. 2009 Mar 1;102(4):1182–1196.

Table E1: Main features of the proposed method

Expression host	Expi293F™ (human origin, suitable for high density and serum free culture conditions)
Vector	Dual system with monocistronic UCOE-vectors (strong CMV promoter for high expression, UCOE sequence able to prevent promoter silencing)
Expression system	Stable
Vessel and physical conditions	Shaking flasks (125 rpm), 37 °C, 8%CO_2
Medium	Expi293™ Expression Medium, serum free
Yield	up to 87 mg/L
Volume	Suitable for small and upscaled cultures (from 15 to 300 mL)
Cell density	up to $11x10^6$ cells/mL
Time to setup the stable platform	~ 50 days
Time for antibody expression	24 hours (2 mg in 25 ml culture)
Cost for the setup of the stable platform	PIPE cloning, adhesion based and small volume stable transfection with Lipofectamine® 2000 and FACS based screening of clones, make the development of the stable platform cost-effective compared to the production of antibody using transient transfections
Cost for antibody expression	High yield in a small volume of Expi293™ Expression Medium (up to 87 mg of IgE for 1 L of medium)

Table E2: PIPE cloning primers

PRIMER NAME	5'-3' SEQUENCE
Isolation of ε heavy chain	
H F	AGTTAACGGCCGGCCATGGACTGGACCTGGAGGATCCTC
E R	TGCAGCTCGAGTCATTTACCGGGATTTACAGACACCGCT
Isolation of κ light chain	
K F	AGTTAACGGCCGGCCATGTTGCCATCACAACTCATTGGG
K R	TGCAGGTCGACCTAACACTCTCCCCTGTTGAAGCTC
UCOE® vector linearization in 3 segments (U1, U2, U3)	
U1 F	GTCGACCTGCAGGCATGCAAG
U1 R	CGTTGTCAGAAGTAAGTTGG
U2 F	CCAACTTACTTCTGACAACG
U2 R	AGCACTAACGAAGTAGAGGG
U3 F	CCCTCTACTTCGTTAGTGCT
U3 R	GGCCGGCCGTTAACTTAACTAAC

Table E3: PIPE cloning PCR conditions

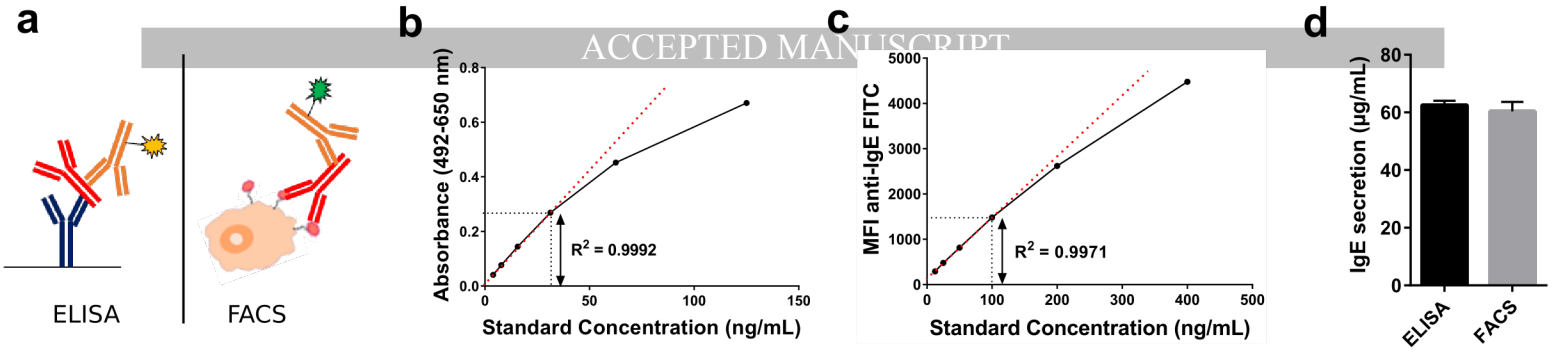
Temperature (°C)	**Time (s)**				
	ε heavy chain	**κ light Chain**	**U1**	**U2**	**U3**
98	10	10	10	10	10
98	1	1	1	1	1
60	5	5	5	5	5
72	23	9	43	42	34

Figure E1. Detection of functionally-active antibodies in culture supernatants by flow cytometry. (**a**) Schematic depicting the principle for antibody detection in cell culture supernatants by classical sandwich-ELISA (ELISA) and by our novel cytofluorimetric based assay (flow cytometry). (**b,c**) Standard curves showing the ranges of linearity (dotted lines) for the ELISA (**b**), and for the flow cytometry method (**c**). (**d**) Comparison between IgE concentrations calculated with ELISA and flow cytometry methods. Error bars represent s.e.m. of three independent experiments. **(e**) Screening of clones transfected with pVITRO1-vector or UCOE-vector system graph representing secreted anti-CSPG4 IgE detected with the flow cytometry method. Clones secreting between 2 and 4 μg/mL of anti-CSPG4 IgE were considered medium expressing, and the ones that produced above 4 μg/mL were considered high expressing. Right panel summarizes absolute numbers and percentages of different expression levels of antibody.

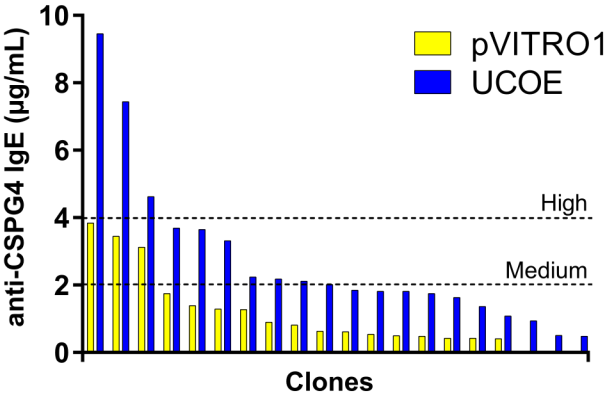
Figure E2. Culture medium conditions for optimal antibody production. To optimize antibody production Expi-CSPG4-IgE cells were cultured in different conditions and IgE secretion and cell viability were monitored daily. (**a**) Secreted IgE and viable cells in cultures seeded at $0.5x10^6$ cells/mL in fresh medium. (**b**) Antibody-specific productivity calculated from (a). Graphs in (a,b) represent one of two independent experiments. (**c**) Secreted IgE (left panel) and viable cells (right panel) in cultures seeded at $5x10^6$ cells/mL in fresh or metabolized medium. Secreted IgE are normalized on secreted IgE at day 0. Data represent mean ± SEM of four independent experiments.

Figure E3. Analysis of the data reported in Figure 1e. Bar chart representing the average yield per passage (mg/L) (**a**), or the average yield per day (mg/day) (**b**). Data represent mean ± SEM of four independent experiments.

Figure E1



e



	pVITRO1	UCOE
Positive clones	17/25 (68%)	20/25 (80%)
Medium expression (2 to 4 µg/mL)	3/17 (17.6%)	6/20 (30%)
High expression (more than 4 µg/mL)	0/20 (0%)	3/20 (15%)

Figure E2

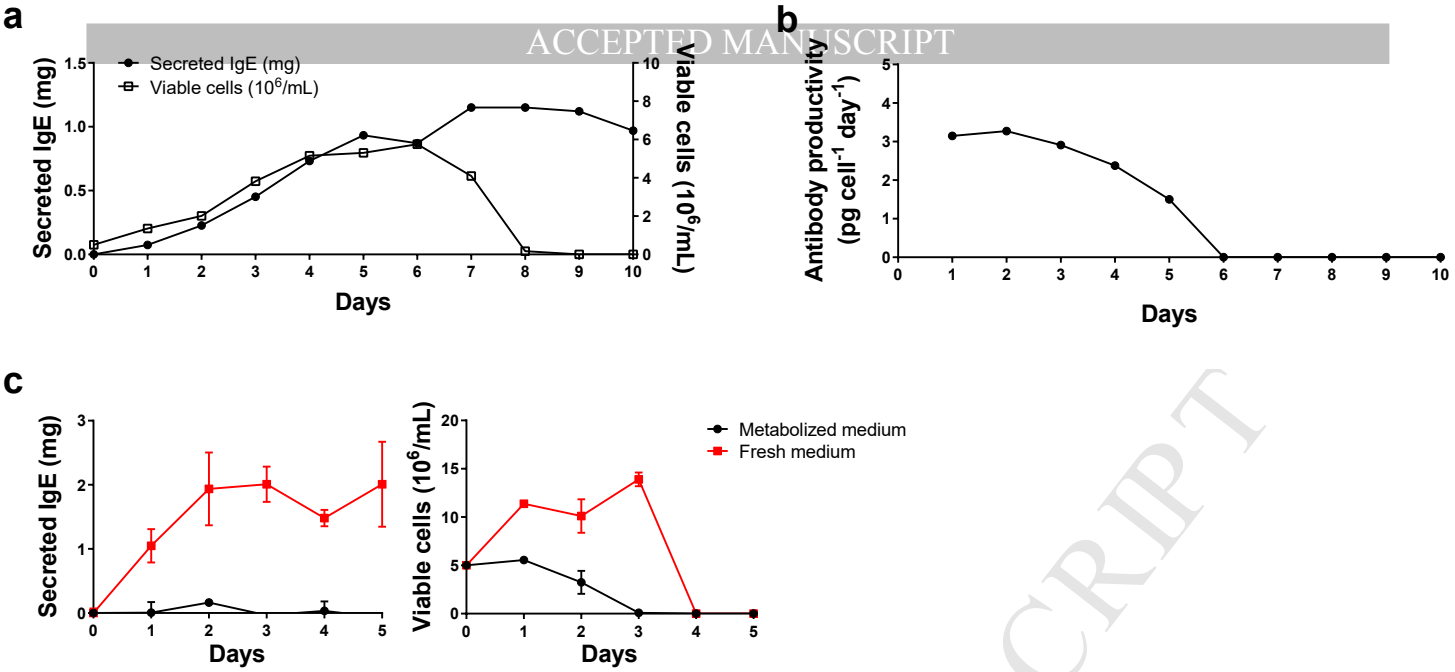


Figure E3